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An Enzyme-Linked Immunosorbent Assay (ELISA) for Trinitrotoluene (TNT) Residue on Hands

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ABSTRACT: An enzyme-linked immunosorbent assay (ELISA) developed for the detection of trinitrotoluene (TNT) in munitions wastewater has been adapted to the detection of TNT residue on hands following contact. Using the procedure developed, as little as 50 pg of TNT could be detected. Accounting for sample size and dilution, the 50 pg equates to 15 ng of TNT recovered from the hands. Following contact with TNT, amounts ranging from 53 ng to more than 1500 ng were recovered from hands. The monoclonal anti-TNT antibodies showed no cross-reactivity with several other explosives or common contaminants. These preliminary results indicate promise for the development of a simple-to-use, immunoassay-based field test kit for TNT and, ultimately, other explosives.

KEYWORDS: forensic science, trinitoluene (TNT), immunoassay, enzyme-linked immunosorbent assay (ELISA), monoclonal antibody

Increased terrorist activity in recent years has prompted the need for new and improved methods for the detection and identification of explosives. The goal of this research was to adapt an enzyme immunoassay developed for trinitrotoluene (TNT) in munitions wastewater to forensic science use. The use of immunoassays in forensic science is typically associated with urine drug testing and serological examinations. An excellent review of enzyme immunoassay techniques and theory has been published [*1*]. This current study deals with the development of an appropriate sampling procedure for TNT on hands. In addition, the sensitivity and selectivity of the assay was evaluated.

The persistence of military and commercial explosives on hands following contact is well documented [*2–5*]. Test kits for explosives using modified Greiss reagents, have been used to detect nitro-containing explosives on hands and surfaces [*6,7*]. Although sensitive to nanogram amounts of explosives, such nonspecific chemical screening tests are subject to potential cross-reactivity or interference from nonexplosive substances such as synthetic nitro musks [*8*]. For this reason, a well-trained user of field test kits will

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have the results confirmed by more specific laboratory tests, such as gas or liquid chromatography or mass spectrometry.

In order to improve the specificity of the screening tests, we chose to undertake the evaluation of a new and powerful method of detecting explosives, an enzyme-linked immunosorbent assay (ELISA) which uses monoclonal antibodies for TNT.

This research adapted anti-TNT monoclonal antibodies and an ELISA procedure developed by Westinghouse Bio-Analytical Systems (WBAS) (Rockville, Maryland) for the detection of TNT in munitions wastewater. The immunoassay procedure involves precoating the plate with a TNT-protein conjugate that adheres to the well surface. Competitive inhibition occurs when samples containing TNT are incubated with mouse anti-TNT antibodies. The antibodies can bind either to the free TNT in the samples or to the TNT bound to the surface of the well. After an equilibrium is reached, the plate is washed and the antibodies bound to the free TNT are washed away, leaving the antibody bound to the immobilized conjugate. Therefore, the amount of antibody bound to the well is inversely proportional to the TNT concentration present in the sample. The greater the concentration of TNT in the sample, the less monoclonal antibody is available to the immobilized TNT conjugate.

The plate is then incubated with goat anti-mouse (μ-chain specific)-alkaline phosphatase conjugate, which binds only to the surface-bound mouse monoclonal anti-TNT antibodies. After the plate is washed, the enzyme substrate is added. The surface-bound enzyme-antibody conjugate converts the colorless substrate to a yellow product. The color is directly proportional to the amount of TNT monoclonal antibody bound in each well and, therefore, is inversely proportional to the amount of TNT in the sample.

Materials and Methods

TNT ELISA Procedure

Ninety-six well, flat-bottom, high-binding microtiter plates (Costar, Cambridge, Massachusetts) were coated with reconstituted Westinghouse Bio-Analytical Systems (WBAS) TNT coating antigen (100 μL/well) and incubated at 37°C for 2 h. The coating antigen was reconstituted in 15 mL of phosphate-buffered saline (PBS) [consisting of 85 g of sodium chloride (NaCl), 2.0 g of monobasic potassium phosphate (KH_2PO_4), and 11.4 g of dibasic potassium phosphate (Na_2HPO_4) in 10 L of deionized water, pH 7.40]. The plate was then washed five times with PBS-T (5 mL of Tween-20 in 10 L of PBS and slapped firmly on an absorbent towel to remove excess PBS-T. Fifty microlitres of sample and TNT standards, ranging in concentrations from 0.33 to 100 ppb, were loaded in triplicate into the wells.

Six wells were reserved for controls. The three negative antibody control wells received 100 μL of the dilution buffer [8.5 g of NaCl, 0.2 g of KH_2OH_4, 1.14 g of Na_2HPO_4, and 1.9 g of ethylenediaminetetraacetic acid (EDTA) in 1 L of deionized water, pH 7.40] in place of the monoclonal anti-TNT antibody. These wells should exhibit little or no color development. To three negative TNT controls, 50 μL of dilution buffer was added in place of the sample. These wells received no TNT and should show maximum color development (no inhibition). The anti-TNT monoclonal antibody was reconstituted in 6 mL of PBS-T, and 50 μL was added to all of the wells except those designated as negative antibody controls. The plate was covered and allowed to incubate for 1 h at room temperature. After incubation, the plate was washed five times with PBS-T and firmly slapped dry.

The antibody-enzyme conjugate was reconstituted in 12 mL of PBS-T, and 100 μL was added to each well. The plate was covered and incubated at room temperature for

30 min. After the incubation, the plate was washed with PBS-T and slapped dry as before. Then 100 μL of the phosphatase substrate solution [15 mg of Sigma 104 phosphatase substrate (Sigma Chemical Co, St. Louis, Missouri) in 16.5 mL of diethanolamine buffer, pH 9.60] was added. The plate was read at 405 nm after the positive control had reached an absorbance of at least 0.8 on a Dynatech MR600 microplate reader (Dynatech Laboratories, Inc., Chantilly, Virginia). A typical calibration curve is shown in Fig. 1. A linear response was observed from approximately 1 to 100 ppb TNT, with a correlation coefficient of 0.997. For example, 50 μL of a 1-ppb solution of TNT corresponds to 50 pg of TNT. For a 1:300 dilution this equates to a total of 15 ng recovered from the hands.

Sample Collection and Preparation

The TNT used in these experiments was a ¼-lb (113-g) demolition block of U.S. military TNT. Samples were obtained by immersing the tip of a cotton swab in ethanol and swabbing the subjects' hands. Prior to handling the TNT, the subjects' hands were swabbed. These blank samples should contain no TNT. After handling the stick of TNT for a brief period, the subjects were instructed to rub their hands together. A set of samples were then obtained in the same manner used for the blanks.

After all of the samples had been collected, the cotton from each swab was removed and immersed in a solution consisting of 900 μL of deionized water and 100 μL of the sample buffer (85 g NaCl, 2.0 g KH_2PO_4, 11.4 g Na_2HPO_4, and 19 g EDTA in 1 L of deionized water, pH 6.85). The samples were vortexed for a minimum of 30 s. Initially, the samples were assayed at dilutions ranging from 1:1 to 1:1000 to determine appropriate dilution levels. A dilution level of 1:300 placed most of the samples within the linear portion of the calibration curve. For the most part, the calibration curve was used to ensure that the assay was proceeding correctly. For the qualitative assays, samples falling within the linear portion of the curve were simply called positive (+). Those exhibiting a response consistent with maximum color development (<1 ppb TNT) were called negative (−).

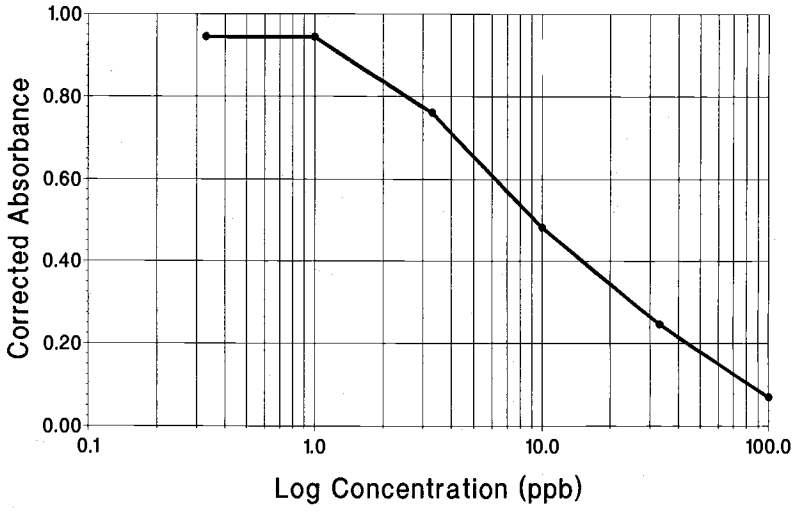

FIG. 1—*Typical TNT assay calibration curve.*

Results and Discussion

Hand Blanks

A series of hand blank samples from six individuals were assayed following the TNT procedure. These individuals had no contact with TNT or other explosives prior to the sampling. Initially, samples were assayed at dilutions ranging from 1:1 to 1:1000 to determine appropriate dilution levels to prevent nonspecific protein binding to the plate. Table 1 shows the results of the hand blank study in which negative TNT controls and negative antibody controls were also analyzed. It was observed that at sample dilutions of 1:300 or greater, a response identical to that of the negative TNT control was obtained.

Recovery from Hands

Five individuals were instructed to handle the ¼-lb (113-g) stick of TNT and rub their hands together. Samples were taken by swabbing the hands prior to and following contact. For the purposes of this initial study, the most efficient means of quantitative recovery from the hands was not investigated. In fact, sample dilutions of 1:300 were required to keep the absorbance in the linear portion of the calibration curve. Duplicate portions of the above samples were provided to WBAS for analysis. These results showed a linear response at sample dilutions of 1:300 and 1:900. Table 2 shows the semiquantitative results from this assay. Accounting for sample size and dilution, the amounts of TNT recovered from the hands ranged from 53 ng to more than 1500 ng.

Effect of Hand Washing

The effect of hand washing after contact with TNT is shown in Table 3. In this test, the subjects handled TNT and rubbed their hands together as before. The first washing was with water alone, followed by three washings with soap and water. After each washing the subjects' left and right hands were sampled.

The samples taken immediately after washing with water were positive for TNT. Samples taken after washing with soap and water were negative. While not all soaps may be effective and the amount and method of hand washing can vary greatly among individuals,

TABLE 1—*Hand blank results at 1:300 dilution.*

Sample	*n*	Absorbance	Standard Deviation
Negative antibody controls	3	0.007	0.002
Negative TNT controls	3	0.828	0.061
Hand blanks	12	0.821	0.058

TABLE 2—*Amounts of TNT recovered from hands.*

Individual No.	Left Hand, ng	Right Hand, ng
1	53	300
2	165	180
3	900	>1500
4	225	160
5	135	85

TABLE 3—*Effect of washing on TNT concentration on hands.*

Sample	Results, L/R
Hand blank	−/−
After handling TNT	+/+
After washing with water	+/+
After washing with soap and water	−/−
After third washing	−/−
After fourth washing	−/−

these results suggest that water alone may not remove all the TNT residue from hands but thorough washing with soap and water may.

Cross-Reactivity with Other Explosives

In order to determine the specificity of the monoclonal anti-TNT antibody, a series of experiments were undertaken to evaluate the cross-reactivity with other explosives under similar handling conditions. As before, left- and right-hand blank samples were taken from the six subjects. Each subject handled one of the explosives listed in Table 4 and was instructed to rub his or her hands together. Left- and right-hand samples were taken again. The cotton was removed from the swabs and added to the water/sample buffer solutions. All of the samples were diluted 1:300 and assayed according to the procedure described above. All the hand blanks produced a negative response. No cross-reactivity was observed between the monoclonal anti-TNT antibody and the six explosives tested.

Contaminants

Musks, as well as a number of other chemicals, are known to present potential interferences with chemical or instrumental means of explosive detection. For example, synthetic nitro musks are similar in chemical structure to TNT and are common fragrance additives in toiletries. To determine their effects on the assay, 100 μL of two undiluted musk perfumes were added directly to the microtiter plates in triplicate. This large concentrated sample of musk is likely to be much more than would be obtained from a hand swab. In these cases, some inhibition of color development was observed. This may be due to partial stripping of the coating antigen from the plate or binding to the monoclonal anti-TNT antibody. Further studies are needed to elucidate the exact mechanism of this interference. However, as shown in Table 5, when diluted 1:300 or greater the samples were observed to be negative. Gasoline, hand lotion, and mouthwash were also

TABLE 4—*Cross-reactivity with other explosives recovered from hands.*

Sample	Results, L/R
Semtex	−/−
Atlas 7-D	−/−
C4	−/−
Flex-X	−/−
Unigel	−/−
PETN	−/−

TABLE 5—*Response of TNT assay in presence of contaminants at a dilution of 1:300.*

Sample	Alone (L/R)	With TNT added
Musk Perfume No. 1	−/−	+
Musk Perfume No. 2	−/−	+
Hand lotion	−/−	+
Mouthwash	−/−	+
Gasoline	−/−	+
Smokeless tobacco	[a]	[a]

[a]Sample was cloudy upon addition to well.

applied to hands and recovered using the prescribed method. No effect on the assay was observed for these items. A small piece of wintergreen smokeless tobacco was extracted and added directly to the well. The extract was visibly cloudy, thus affecting the absorbance measurement.

Another test was performed to determine if the diluted contaminant would prevent a positive response in the presence of TNT. In this test, a 100-ng TNT standard was added to the wells in addition to the diluted contaminant. The assay showed a positive response for TNT in the presence of the musks, hand lotion, gasoline, and mouthwash. As previously mentioned, the tobacco extract was cloudy. Filtration may be necessary for samples containing particulate matter.

Conclusions

These preliminary results indicate that an enzyme immunosorbent assay developed for the detection of TNT in munitions wastewater was successfully adapted to the detection of TNT on hands. These results indicate that the mouse monoclonal anti-TNT antibody for TNT is specific for TNT and shows no cross-reactivity with those explosives or interferents tested in this study. The assay was capable of detecting as little as 50 pg of TNT.

Although the application of enzyme immunoassay to the analysis of explosives is in its infancy, these results indicate that it should be possible to reconfigure the laboratory assay into a simple immunoassay-based field test kit. The resulting monoclonal antibody-based test kits should have improved specificity over current test kits. Finally, the development of monoclonal antibodies to other explosives should lead to future developments in this new area of explosives detection.

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